Cancer is probably man’s most dreaded disease in the modern world and affects almost one in three people at some point time in their life time. For most individuals it is a disease of old age and occurs by chance. Cancer is a common disease, which is second only to diseases of heart in terms of mortality rate in adults. In spite of spending billions of dollars for cancer treatment and research, the cancer statistics have not come down. The expectation of the National Cancer Institute, USA to accomplish 50% of cancer patients to be cured by the end of year 2000 was not successful owing to various reasons mainly due to the toxicity of isolated compounds and synthetic drugs. Further, the treatment of solid tumor still remains an elusive and distant goal (Schwartsmann et al., 1988). Among the several treatment modalities available for the treatment of cancer, surgery, radiotherapy and chemotherapy still continue to be the most important (DeVita et al., 2005).

The aim of radiotherapy is to deliver as high a dose as possible to destroy the malignant tissues without causing excessive injury to the surrounding healthy tissue (Robinson, 2008). Although this mode of therapy is often effective, its success is limited by inherent resistance of certain tumors, presence of hypoxic cells and heterogeneous population with varying degrees of radio-sensitivity. Also curative doses alone are not tolerable because of normal tissue toxicity. Very narrow molecular differences between the cancer and normal cells make it non-selective therapy (Hendry et al., 2006).

In order to improve the outcome of radiotherapy, several compounds have been evaluated which either sensitize the tumor cells to the effects of radiation (radiosensitizers) or protect the normal cells from the deleterious effects thereof (radioprotectors). Although these studies have yielded many such radiosensitizing chemical agents that have shown potential in the experimental tumor models, the clinical effectiveness has always been elusive. Therefore, attempts were made to improve the therapeutic outcome by combining radiation with conventional chemotherapeutic agents and such efforts have yielded fairly encouraging results (Caffo, 2001; Lawrence et al., 2003; Horsman et al., 2006; Seiwert et al., 2007). Nevertheless, the side effects of such modes of treatment are severe and have often resulted in the occurrence of secondary malignancies (Herskovic et al., 1992; Kirwan
et al., 2003; Sijben et al., 2008; Udagawa, 2009) and therefore research efforts to explore compounds with better chemotherapeutic and/or radiosensitizing potential shall be continued to reduce toxic side effects of combination treatment and thereby improving the outcome of the therapy.

The severe toxic side effect of the conventional chemotherapeutic agents has always been the central problem in the modern day cancer chemotherapy. Any approach that can tackle the toxicity issues without compromising the anticancer potential of compounds could potentially improve the therapeutic outcome in cancer. One such approach that has been found to be largely successful is to make use of the various drug delivery platforms to target (either actively or passively) the entrapped compound to the tumor tissue thereby restricting the entry of the drug into unintended organs and reducing the associated toxicity. Among the various drug delivery platforms available to date, liposomes have gained the most prominence. Apart from being biodegradable/biocompatible, they capable of encapsulating both hydrophilic and hydrophobic drugs and also target encapsulated drug to the tumor tissue (Sharma and Sharma, 1997). Also, they are regarded as very flexible, in a way that their surfaces can be easily modified with a variety of functional moieties such as polyethylene glycol (PEG) and targeting ligands (Moghimi and Szebeni, 2003).

However, no unified treatment for cancer has emerged so far which underscores the importance of discovering compounds with anticancer and radiosensitizing potentials with acceptable toxicities. With that perspective, researchers from around the world have been engaged in the search for new anticancer compounds with radiosensitizing properties. Herbs offer a vast source of new chemicals and some of them are structurally so complex that they cannot be synthesized even with the advanced technology at our disposal (Balunas and Kinghorn, 2005; Koehn and Carter, 2005). The herbal drugs have gained attention and popularity because of their negligible toxicity and possibly with a ray of hope that they may replace some of the available antineoplastic drugs that are highly toxic.

Naturally occurring quinones have been extensively studied for their potential as anticancer agents and such efforts have yielded several clinically useful anticancer
drugs including mitomycin C, doxorubicin, mitoxantrone, siantopin etc (Kim et al., 2006). Besides, several other plant derived quinones (including plumbagin, beta-lapachone, menadione etc) have also shown promising anticancer effect against various cancer cell types both in vitro as well as in vivo (Nutter et al., 1991; Devi et al., 1999; Pardee et al., 2002). In addition, they are also known to possess potent radiosensitizing potentials against various tumor models (Taper et al., 1996; Nair et al., 2008).

Juglone (5-hydroxy-1, 4-Napthoquinone), a structural analogue of plumbagin is a pigment that occurs as a natural product in the roots, leaves, nut-hulls, bark and wood of various walnut species belonging to the family Juglandaceae (Botanical Dermatology Database, 1999). The herbal preparations of walnut have been extensively used in folk medicine for the treatment of acne, inflammatory diseases, ringworm, bacterial, viral, fungal infections and also cancer (Duke and Ayensu, 1985; Blumenthal, 1998). Bhargava and Westfall reported the potential of the herbal extract of walnut to suppress the growth of spontaneous mammary adenocarcinoma in swiss albino mice (Bhargava and Westfall, 1968). Although few groups have studied the in vitro cytotoxic activity of juglone against cancer cell lines (Segura-Aguilar et al., 1992; Cenas et al., 2006; Li et al., 2010; Ji et al., 2011), the exact mechanism remains ambiguous. However, the in vivo anticancer potential of juglone has been ambiguous with some earlier reports suggesting potent anticancer properties in vivo (Okada et al., 1967; Sugie et al., 1998; Ji et al., 2009), and other suggesting otherwise (Van Duuren et al., 1978; Monks et al., 1990). Also there are no earlier reports of the radiosensitizing potential of juglone. Further, juglone is reported to possess some normal tissue toxicity including contact dermatitis (Neri et al., 2006). Available literature also suggests that no previous attempts have been made to target juglone to the tumor tissue, aimed at reducing its normal tissue toxicity and improving its anticancer activity.

Therefore the present study was aimed to evaluate the anticancer and radiosensitizing potential of juglone against in vitro and in vivo tumor models. In addition, the present investigation was intended to formulate juglone as sterically
stabilized liposomal forms aiming enhanced antitumor efficacy and reducing the toxicity associated with juglone

AIMS & OBJECTIVES OF THE STUDY

- To study the cytotoxic potential of juglone in vitro against a panel of human and murine tumor cell lines and to understand the mechanisms underlying the cytotoxic potential against B16F1 melanoma cells grown in vitro.

- To evaluate the in vivo anticancer and radiosensitizing potential of juglone against B16F1 melanoma cells grown as solid tumor on C57BL/6J mice and also to evaluate the radiosensitizing potential of juglone against B16F1 melanoma cells grown in vitro.

- To formulate, characterize and optimize sterically stabilized liposomes of juglone with an aim of improving the anticancer efficacy and to reduce the toxicity associated with juglone.

- To comparatively evaluate the optimized sterically stabilized liposomal formulation of juglone against free juglone for the pharmacokinetic, biodistribution, pharmacodynamic as well as toxicity profiles of juglone.

Initial MTT assay studies using a panel of human and murine tumor cell lines revealed MCF7 human breast cancer cells were most sensitive to the cytotoxic effects of juglone with an IC\(_{50}\) value of about 3.8 and 2.7 µM after 24 and 48 h respectively. The second most sensitive cell line was B16F1 mouse melanoma with an IC\(_{50}\) value of 7.5 and 6.9 µM after 24 and 48 h respectively. The other tested cell line (ACHN - human renal carcinoma and A549 – human lung carcinoma) were much more resistant with IC\(_{50}\) values > 10 µM. All the further studies were performed using B16F1 melanoma cells bearing in mind that the in vivo studies were to be performed using these cells. Treatment of B16F1 melanoma cells with juglone resulted in a concentration-dependent decrease in the clonogenicity with a corresponding increase in the lactate dehydrogenase levels. Juglone treatment also caused a significant increase in the genotoxicity as evidenced by the concentration-dependent elevation in the micronucleated binucleate cells as well as olive tail moment (OTM) values. Experiments were designed to evaluate the role of reactive oxygen species in the
juglone induced cytotoxic effect against melanoma cells, where a significant concentration-dependent decrease in the intracellular glutathione levels and an increase in dichlorofluorescein (DCF) fluorescence was observed, which confirmed the ability of juglone to generate intracellular reactive oxygen species. Further, the juglone-induced apoptotic and necrotic mode of cell death was demonstrated by oligonucleosomal ladder formation, microscopic analysis, increase in the hypodiploid fraction (sub Go peak in DNA histogram) as well as increase in percentage of AnnexinV(+)/PI(+) cells analyzed by flow cytometry.

Further studies were designed to evaluate the in vivo anticancer and radiosensitizing potential of juglone against B16F1 melanoma cells growing as solid tumors on the dorsal side of C57/BL6J mice. Initial acute toxicity studies carried out and analyzed by probit method revealed that juglone had LD$_{50(14)}$ value of around 4.2 mg/kg b. wt. when administered intravenously. Subsequently, experiments were carried out based on these acute toxicity studies, wherein 1 mg/kg b. wt. was found to be optimum for anticancer effects. The effect of juglone on the DNA damage levels in the tumor as well as nucleated blood cells of animals treated with optimum dose of juglone was evaluated. A significantly higher DNA damage was observed in the tumor tissue, consistent with the earlier in vitro findings, wherein juglone treatment of B16F1 cells in vitro showed significantly higher OTM values (indicative of increased DNA damage). However, this study also showed significantly higher levels of OTM in nucleated blood cells indicative of potential normal tissue genotoxicity. Further, combining the optimum dose of juglone with radiation (10 and 30 Gy) resulted in a significant reduction in the tumor growth kinetics as evidenced by the volume doubling time as well as growth delay values. In addition, the in vitro radiosensitization studies using clonogenic assay revealed the potential of juglone in augmenting the radiation induced cell kill in B16F1 melanoma cells, where a sensitization enhancement ratio of 1.37 for the combination treatment compared to radiation alone group was observed. Similar effects were also seen in the case of comet assay studies where juglone was found to enhance the radiation induced DNA damage in melanoma cells (evidenced by the OTM values). From the DCFH-DA studies, it was found that treatment of melanoma cells with a combination of juglone
followed by radiation caused significantly higher reactive oxygen species’ (ROS) levels in comparison to independent treatment indicating the role of ROS in the juglone-induced radiosensitization in vitro.

Based on the previous findings described in chapter 4, wherein juglone was found to cause toxicity to the nucleated blood cells and also based on the existing literature on its toxicity profile, attempts were made to formulate juglone as sterically stabilized liposomes (SSL). Initial pre-formulation studies using FTIR revealed that juglone was compatible with all the excipients intended to be used in the formulation. From the solution stability studies, it was found that juglone had pH dependent stability in solution where it was more stable in acidic pH and became more and more unstable as the pH of the solution was increased. Based on these studies, liposomes were formulated by conventional thin film hydration method using acetate buffer (pH 4.0) as the hydration medium. The formulations were optimized for size, zeta potential, polydispersity index, entrapment efficiency as well as in vitro release profiles by altering the cholesterol as well as mPEG2000-DSPE content. The size and entrapment efficiency increased as the concentration of cholesterol increased from 9:0.5:0.3 to 9:3:0.3 (SPC:Cholesterol:mPEG2000-DSPE). Further increase did not cause any improvement in the entrapment efficiency of juglone into the liposomes. From the in vitro release studies, a clear inverse relation between the mPEG2000-DSPE content and % cumulative drug release was seen. From all these studies, the optimized liposomal formulation of juglone (JL9) had a mean particle size of 137.1 nm, zeta potential of -45.7 mV and released about 60 % of the entrapped drug after 24 h. The in vitro cytotoxicity studies of SSL juglone in comparison to free juglone revealed that SSL juglone was more toxic than free juglone against melanoma cells which may be attributed to the improved stability of juglone in the solution when formulated as liposomes. The in vivo evaluation of this optimized formulation may be carried out in comparison to free juglone to evaluate the beneficial effect of formulation on its in vivo behavior.

Initial pharmacokinetic studies revealed that free juglone had a short plasma half life of about 2 h and was rapidly eliminated from the systemic circulation. In contrast, formulation of juglone as sterically stabilized liposomal form significantly
improved the pharmacokinetics with a 12-fold increase in the plasma half-life, 4.5-fold increase in the AUC\(_{0-\infty}\), 10-fold reduction in the renal clearance rates and a 3-fold increase in the mean residence time of juglone. Further, from the biodistribution studies of free juglone, a large accumulation of juglone induced radioactivity in the kidneys was observed indicating that the juglone may be rapidly eliminated through the renal route. Accumulation in other organs was also observed in the following order kidney > liver > heart > spleen ≥ tumor. However, formulation of juglone as sterically stabilized liposomes reduced the accumulation in the kidneys and heart significantly with significantly higher accumulation in the liver, spleen as well as the tumor tissue. This increased tumor accumulation of juglone was further confirmed from the pharmacodynamic studies where liposomal juglone exhibited significantly better anticancer as well as radiosensitizing potential in comparison to free juglone. The toxicity studies were also carried out where we observed significant levels of nephrotoxicity in the free juglone treated group (evidenced by the necrosis of the renal convoluted tubules as well as glomeruli) which significantly reduced in the mice treated with liposomal juglone, which corroborates the findings of biodistribution studies. However, in spite of higher accumulation of liposomal juglone in organs like the liver and spleen, no significant toxicities were seen from histological studies, which may be due to the slow release of juglone from the liposomes. Also the toxicity of juglone to the nucleated blood cells was significantly lower (as evidenced by the OTM values) when administered as sterically liposomal forms as compared to free juglone.

From the present study, following important conclusions can be drawn

1. Juglone exhibited potent cytotoxic potential against both human (MCF7 - breast carcinoma, ACHN – renal carcinoma, A549 – lung carcinoma) as well as murine tumor cells (B16F1 melanoma) \textit{in vitro}.

2. A multi-factorial mechanism involving oxidative stress, cell membrane damage and genotoxic effect may have contributed to the juglone induced apoptotic and necrotic mode of cell death in B16F1 melanoma cells \textit{in vitro}.

3. Juglone treatment at an optimum dose of 1 mg/kg b. wt. significantly reduced the growth of B16F1 melanoma grown as solid tumor on C57/BL6J mice.
4. Juglone treatment resulted in differential genotoxic effect as evidenced by the higher levels of DNA damage in the tumor tissue in comparison to nucleated blood cells.

5. Juglone treatment at an optimum dose of 1 mg/kg b. wt. followed by radiation treatment significantly reduced the growth of B16F1 melanoma cells (in vivo) in comparison to radiation treatment alone, indicating its radiosensitizing potential in vivo.

6. Juglone exhibited radiosensitizing potential in vitro which may be attributed to reactive oxygen species mediated DNA damage and its delayed repair.

7. Juglone formulated as sterically stabilized liposomes exhibited increased cytotoxicity in comparison to free juglone against B16F1 melanoma cells grown in vitro which may be attributed to the improved stability of juglone as liposomal forms.

8. Formulation as sterically stabilized liposomes significantly improved the pharmacokinetic profile of juglone as evidenced by the increased plasma half life, reduced renal clearance and increase mean residence time.

9. Formulation as sterically stabilized liposomes significantly altered the biodistribution pattern of juglone with higher tumor accumulation and reduced accumulation in the kidney.

10. Formulation as sterically stabilized liposomes also resulted in improved anticancer and radiosensitizing potential of juglone against B16F1 melanoma cells grown in vivo.

11. Formulation as sterically stabilized liposomes significantly reduced the nephrotoxicity (as evidenced by the histological observations) as well as DNA damage levels in nucleated blood cells (as evidenced by the lower OTM levels) associated with free juglone.

Based on these studies, liposome based drug delivery platforms may offer significant advantage in the intravenous delivery of hydrophobic anticancer molecules like juglone. However, more elaborate pre-clinical studies need to be carried out against an array of human tumor models growing as xenografts in nude mice and establish the safety profile before advocating clinical evaluation.
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